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INTRODUCTION:

This report details our progress to date describing the inhibition of the transcription factor, Nuclear Factor kappa B (NFκB) with parthenolide. To date we have shown that NFκB is constitutively active in prostate cancer cell lines and endothelial cells and that NFκB DNA binding is inhibited by parthenolide. Moreover, we have found that NFκB is over-expressed in human prostatectomy specimens at both the prostatic intraepithelial neoplasia and invasive adenocarcinoma stages. With the use of cDNA array technology we have shown that multiple genes associated with the hallmarks of cancer and that are under NFκB control are decreased when cancer and endothelial cells are treated with parthenolide. We have subsequently shown that parthenolide is able to decrease cancer cell proliferation and enhance the cytotoxic effect of taxanes *in vitro*. Finally, we have shown that parthenolide is bioactive *in vivo* as it is able to inhibit angiogenesis as a single agent, enhance the cytotoxic effects of docetaxel and restore sensitivity of the hormone independent cell line, CWR22Rv1, to the anti-androgen, bicalutamide.

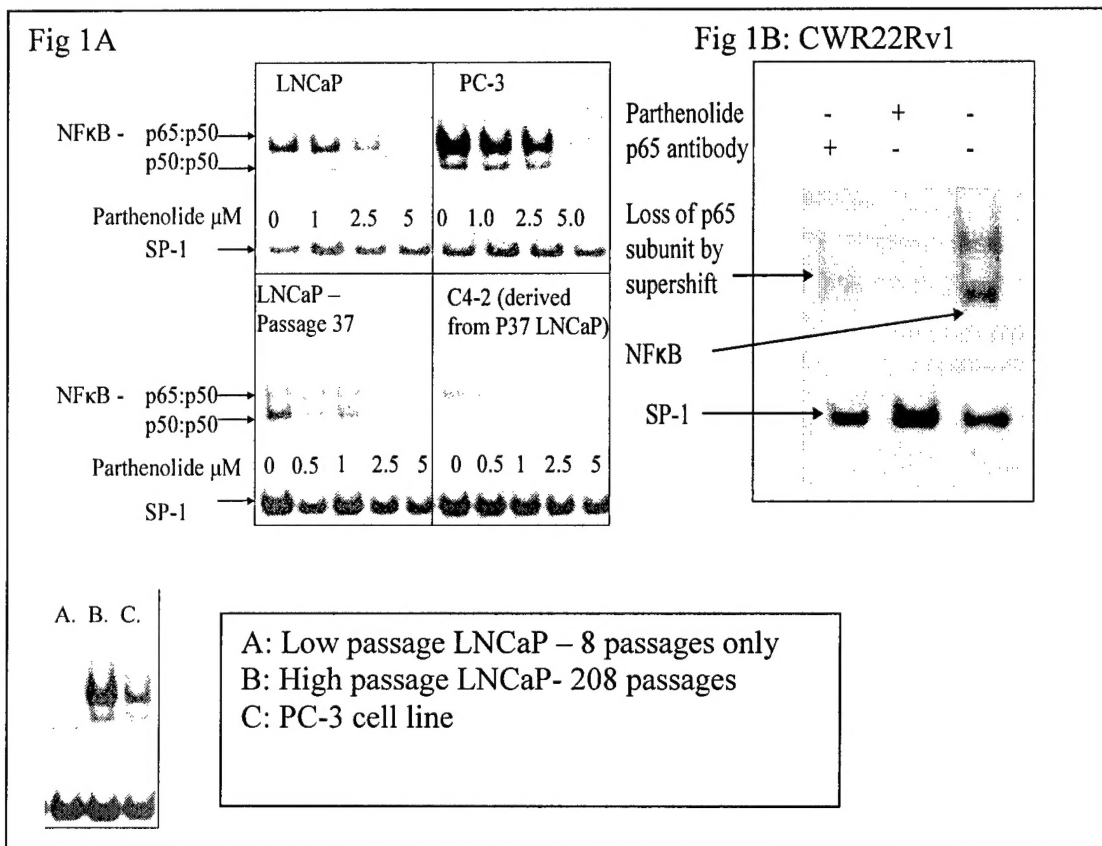
BODY:

Task 1. To identify the relative amount of the NFκB p65:p50 heterodimer and the genes increased in response to greater NFκB activity in hormone refractory cells (Months 1-12):

a) Culture hormone dependent(LNCaP) and hormone independent cells (C4-2)and perform a gel-shift analysis (Months 1-9).

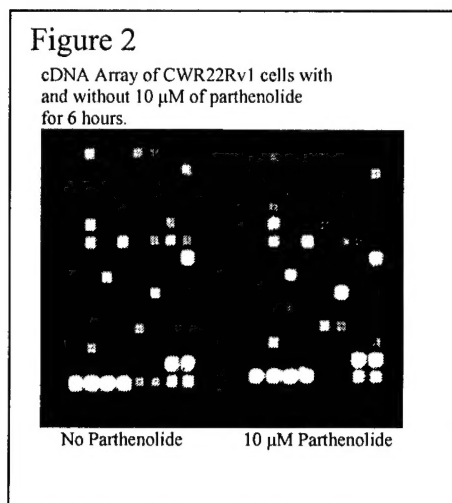
b) Perform the gel shift assay with cells treated with parthenolide (Months 1-9).

A notable function of parthenolide described by several investigators is its ability to inhibit NFκB¹⁻⁴. NFκB is a collection of dimers composed of members of the Rel family of transcription factors. The most active NFκB dimer is the p65:p50 heterodimer. NFκB dimers are bound to inhibitory IκB proteins in the cytoplasm and are released from IκB after phosphorylation of IκB by IκKinase (IKK) by proteosome mediated degradation of IκB. The release of NFκB and subsequent binding to DNA occurs in response to a variety of stimuli including cytokines such as Tumor Necrosis Factor (TNF) and interleukin-1 (IL-1) which are found in the microenvironment of many cancers⁵. We have found *in vitro* that NFκB DNA binding is present in all prostate cancer cell lines evaluated (Figure 1). The proportion of the p65:p50 heterodimer to p50:p50 homodimer is lowest in the LNCaP cell lines that have undergone the least number of passages in culture compared with (i) the LNCaP cell lines that have undergone a higher number of passages and (ii) the hormone independent cell lines – C42 and PC-3 (Figure 1A). Also of note is that parthenolide is able to inhibit the NFκB DNA binding in a dose dependent manner starting at 0.5 μM. EMSA also showed that NFκB DNA binding is present in the androgen independent prostate cancer cell line, CWR22Rv1, and supershift confirmed the presence of the active subunit, p65. NFκB DNA binding was again inhibited by parthenolide (Figure 1B).



c. Perform a ribonuclease protection assay to analyze for altered levels of gene expression (Months 6 to 9).

At the time writing this grant application we proposed to perform a global analysis of parthenolide's ability to suppress genes under NFκB control using the ribonuclease protection assay (RPA). We have opted to perform the evaluation



using a NFκB Superarray instead of the RPA as it assesses more genes under NFκB control and permits quantification of changes by image analysis. The rationale for this evaluation is the following: Genes activated by NFκB play a central role in many of the hallmarks of cancer including: *invasion* (IL-6, matrix metalloproteinase 9; *angiogenesis*: (IL-8, vascular endothelial growth factor [VEGF]); *organ specific homing of metastatic cells* (CXCR4); and *evasion of apoptosis*: (cIAP-1, c-IAP-2, TRAF-1, TRAF-2, Bfl-1/A1, Bcl-X_L and manganese superoxide dismutase⁶⁻¹¹). The relative expression level of 96 transcripts under NFκB control was determined before and after parthenolide treatment using chemiluminescence and the GEArray

Analyzer. All results were normalized by adjusting for the signal derived from GAPDH and β-actin spots. The change in a given gene transcript from one experiment was estimated by comparing the signal intensities of paired specimens. Genes that had at least a two-fold decrease after 6 hours of treatment in both experiments and the average of the two was greater than four-

fold were arbitrarily considered to show a magnitude and consistency in effect between experiments to support the hypothesis that genes under NF κ B control are present in prostate cancer cells and provide relevance to the electromobility gel shift findings above. The 23 genes that met these criteria and are under the transcriptional control of NF κ B¹² are detailed in Table 1. It is of note is that many of the genes associated with the hallmarks of cancer¹³ were decreased. Specifically, genes associated with evasion of apoptosis, TNF receptor associated factor-1 (TRAF-1) and TRAF-5 were decreased. Also genes associated with maintaining cell-cell adhesion and thus preventing metastasis (ICAM-2, ICAM-5 and VCAM) and genes associated with "unlimited" growth (c-myc) were also decreased.

Gene	Experiment 1*	Experiment 2*	Average of 1&2*
Granulocyte-Macrophage Colony stimulating factor	3.24	9.88	6.56
Intercellular adhesion molecule 2	6.1	6.81	6.455
Intercellular adhesion molecule 5, telencephalin	5.25	2.98	4.115
Interferon, alpha 1	4.67	6.8	5.735
Interferon, beta 1, fibroblast	6.68	8.75	7.715
Interleukin 12B, p40	3.74	5.36	4.55
Interleukin 1, alpha	2.57	8.7	5.635
Interleukin 1, beta	4.86	5.65	5.255
Interleukin-1 receptor type II	8.43	5.63	7.03
Interleukin-1 receptor-associated kinase (IRAK) mRNA	3.99	8.25	6.12
Lymphotoxin-alpha (TNF superfamily, member 1)	5.47	4	4.735
Transforming growth factor beta-activated kinase 1	3.28	9.04	6.16
Protein kinase, mitogen-activated 3 (MAP kinase 3; p44)	5.75	6.07	5.91
v-myc avian myelocytomatosis viral oncogene homolog (c-myc)	9.6	7.36	8.48
Neural cell adhesion molecule 1	6.45	4.39	5.42
Orosomucoid 1 (ORM1, alpha-1 acid glycoprotein)	4.67	4.97	4.82
TNF Receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP)	9.55	7.81	8.68
Transforming growth factor beta-activated kinase-binding protein 1 (TAB1),	8.37	5.04	6.705
Toll-like receptor 10 (TLR10),	7.11	2.09	4.6
Tumor necrosis factor (TNF superfamily, member 2)	5.42	7.22	6.32
TNF receptor-associated factor 1 (TRAF-1)	6.02	7.18	6.6
TNF receptor-associated factor 5 (TRAF-5)	8.06	7.7	7.88
Vascular cell adhesion molecule 1	7.31	9.99	8.65

*Fold decrease

d. Correlate the level of gene expression with changes in the corresponding protein (Months 9 to 12)

This experiment is being undertaken at the time of writing this report. The delay is because of the time to validate the Superarray assay in our laboratory. As can be seen below we have been

able to adapt our timeline and contemporaneously complete most of Tasks 2 and 3 which were to be done in year 2 and 3. As such we are still on schedule to complete the work in the timeframe of the award. Western blot analysis of relevant proteins under NF κ B control after time course treatments with parthenolide will also be done to assess for changes (eg TRAF-1, cyclin D1, MnSOD).

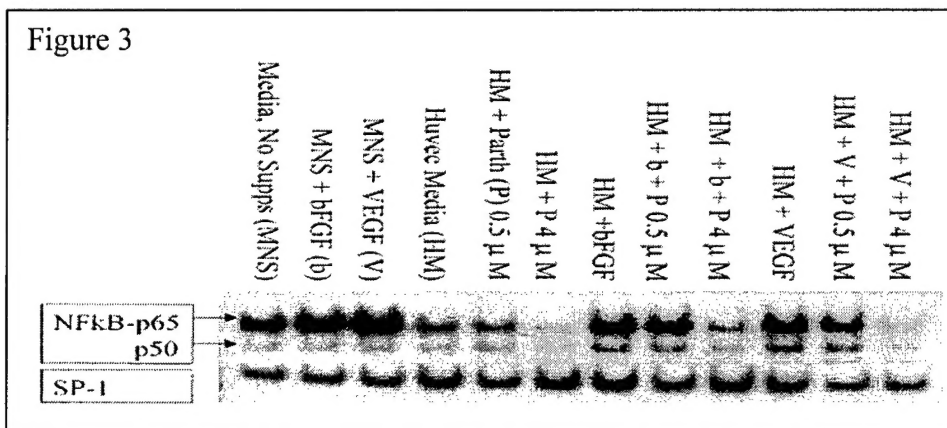
Work to be done in final year:

- 1) Western blot analysis of relevant proteins under NF κ B control
- 2) Compare the gene expression of NF κ B LNCaP cells with low (passage 12, with very little NF κ B DNA binding with high passage (passage 209) and assess their sensitivities to hormonal therapy.

Task 2. To identify the growth factors that increase NF κ B activity in human umbilical venous endothelial cells and the genes that are increased in response to NF κ B activity (Months 13 to 18):

- a). Culture HUVECs and perform a gelshift analysis with solvent control, VEGF and bFGF (Months 13 to 18).
- b). Perform the gel shift assay with cells treated with low dose parthenolide (Months 13 to 18).

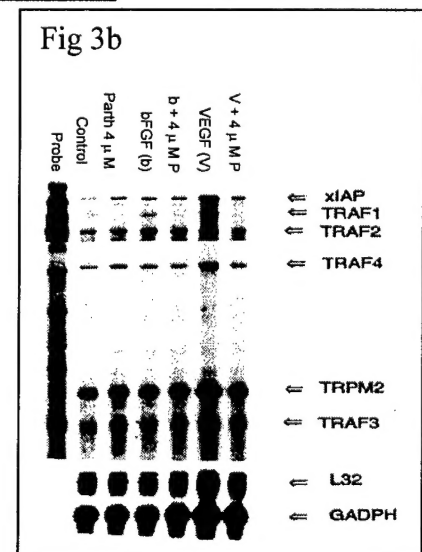
As can be seen in figure 2, NF κ B DNA binding is present in HUVECS and can be increased by VEGF and bFGF and suppressed by parthenolide.



- c). Perform a ribonuclease protection assay to analyze for altered levels of gene expression (Months 13 to 18).

At the time of writing this grant application we proposed to perform a global analysis of parthenolide's ability to suppress genes under NF κ B control using the ribonuclease protection assay (RPA). We did perform this as shown in Figure 3. As one can see, bFGF and VEGF increase TRAF-1 and this is decreased with parthenolide.

We have again also opted to perform the evaluation using a NF κ B Superarray instead of the RPA as it assesses more genes under



NF κ B control and permits quantification of changes by image analysis. The rationale for this evaluation is as described for the prostate cancer cells. This global analysis reveals that parthenolide is able to decrease multiple genes under NF κ B control.

d. Correlate the level of gene expression with changes in the corresponding protein.

This experiment is being undertaken at the time of writing this report. The delay is because of the time to validate the Superarray assay in our laboratory. As can be seen below we have been able to adapt our timeline and contemporaneously complete most of Tasks 2 and 3 which were to be done in year 2 and 3. As such we are still on schedule to complete the work in the timeframe of the award.

Gene	Experiment 1*	Experiment 2*	Average of 1&2*
ELK3, ETS-domain protein (SRF accessory protein 2)	5.566	4.505	5.035
Fas (TNFRSF6)-associated via death domain (FADD)	2.072	4.947	3.509
Homo sapiens interferon, alpha 1	2.258	4.249	3.254
Interferon, beta 1, fibroblast	6.797	9.270	8.033
Interleukin 12B, p40	4.530	5.469	5.000
Interleukin 1, alpha	3.397	6.340	4.868
Interleukin-1 receptor-associated kinase (IRAK) mRNA	9.672	8.952	9.312
Interleukin-1 receptor-associated kinase 2 (IRAK2),	9.577	9.601	9.589
v-jun avian sarcoma virus 17 oncogene homolog	6.262	8.579	7.420
Myeloid differentiation primary response gene	5.274	2.428	3.851
Homo sapiens nuclear factor related to kappa B binding protein (NFRKB)	8.932	3.384	6.158
Serum amyloid A1	8.182	3.231	5.707
Homo sapiens TANK-binding kinase 1 (TBK1)	5.575	2.032	3.803
Homo sapiens toll-like receptor 5 (TLR5),	7.967	5.902	6.935
Homo sapiens toll-like receptor 6 (TLR6)	7.206	2.960	5.083

Work to be done in the final year

1) Western blot analysis of relevant proteins under NF κ B control with and without bFGF and in another experiment with and without VEGF. Time course treatments with parthenolide will also be done to assess for changes in relevant proteins (eg TRAF-1, cyclin D1, MnSOD).

Task 3. Evaluate the anti-cancer properties of parthenolide.

a). Perform *in vitro* cell proliferation assays of parthenolide in LNCaP, C4-2 and HUVECs as a single agent in combination with docetaxel and 2-methoxyestradiol and charcoal stripped serum (Months 19 to 22)

Figure 4

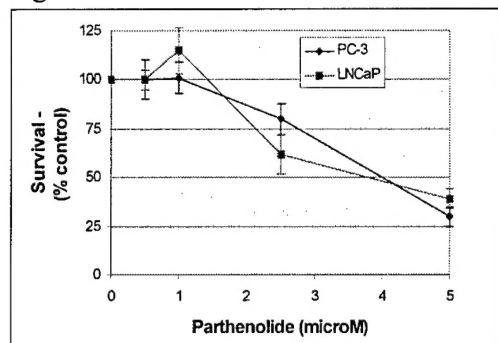
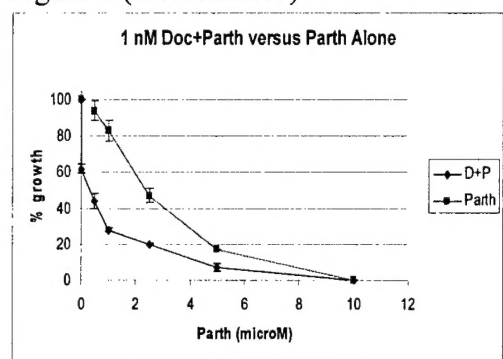


Figure 5 (CWR22Rv1)



We have shown that parthenolide is able to decrease prostate cancer cell growth *in vitro*. Figure 4 shows the results of an MTS-PMS assay of LNCaP and PC-3. In the original grant application we proposed to evaluate LNCaP and C42 in the combinations described above. We subsequently had problems with the media for the C42 cell line and got unreliable results. We have switched to the CWR22Rv1 cell line for the experiments. To assess the expansion of cell number in this cell line we performed clonogenic assays as it was not reliably assessed with the MTT-PMS assay. This change also complimented the *in vivo* work described below as this cell line has been growing reliably in both the *in vitro* and *in vivo* settings. As shown in Figure 5 we have shown that parthenolide significantly increases the cytotoxic effectiveness of docetaxel with 1 nM docetaxel alone decreasing the formation of clones by 40% and parthenolide at 1 μ M decreased clone formation by only 20% however, the combination at these doses decreased the number of clones by 75%. Our previous report has detailed that parthenolide is able to decrease HUVEC proliferation with an IC₅₀ of 7.5

μ M and capillary formation with an IC₅₀ of 4 μ M.

b). Perform *in vivo* experiments with 20 mice in each cohort of parthenolide, docetaxel, 2-methoxyestradiol and parthenolide + docetaxel and parthenolide + 2 methoxyestradiol and parthenolide + castration (Months 22 to 33).

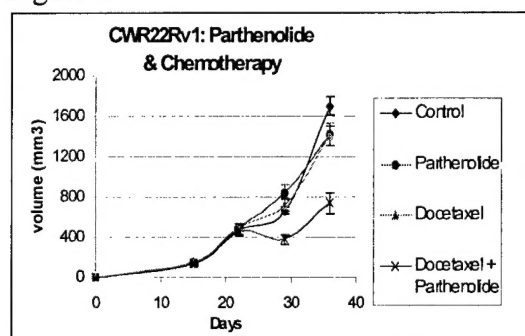
To facilitate translation of our *in vitro* findings into representative animal models, we determined the plasma concentration of parthenolide in mice following a two week dosing regimen. Parthenolide was administered via oral gavage at dose ranges from 0.4 up to 40 mg/kg/day. One hour post-gavage, we measured plasma concentrations of 112 nM at the 4mg/kg dose and 170 nM at the 40mg/kg dose. Parthenolide is a very lipophilic molecule and studies have shown that the systemic concentration can underestimate the cellular concentration of such lipophilic drugs^{14,15}. However, no overt acute toxicity was evident in any of the animals and we therefore pursued the 40 mg/kg/day dose regimen in the following *in vivo* tumor models.

Of common concern to all cancer investigators is the difficulty in relating the available tumor models to the human disease. Therefore, we selected a model based on its clinical relevance and resistance to chemotherapy and hormonal therapy. Hence we used the human derived androgen resistant cell line, CWR22Rv1 in the flank of the subcutaneous tissue of male mice¹⁶. We initially performed experiments with C-42 implanted into the tibia of male mice, however, we were not able to measure tumor volume reliably and we switched to CWR22Rv1 in the flank.

To date we have compared the activity of parthenolide with both docetaxel and the anti-androgen, bicalutamide. Docetaxel was chosen as a point of reference since it is one of the most active drugs in the clinic for prostate cancer. Bicalutamide was chosen with the intent of determining whether parthenolide could restore some degree of sensitivity to hormonal therapy to this cell line that grows in castrate mice¹⁷. It also needs to be pointed out that therapy was started 14 days after tumor implantation so the tumors would be established and undergoing exponential growth. This is a more “rigorous” model rather than commencing therapy when the tumors are only starting to adapt to their new environment and better represents the clinical setting of macroscopic metastatic disease. As such if activity is observed, we would have a more reliable read-out.

Parthenolide with weekly docetaxel in a prostate cancer heterotopic model: The treatment schedule employed was: 1) 40 mg/kg of parthenolide per day in 100 μ L of 13% alcohol via oral gavage, 2) 5 mg/kg of intraperitoneal (i.p.) docetaxel in 13% alcohol weekly or, 3) the combination of docetaxel and parthenolide.

Figure 6:



Appropriate vehicle controls were used (oral daily and i.p. weekly). To extend the above findings beyond one cancer cell line and tumor type, we established mice bearing the hormone independent prostate cancer cell line CWR22Rv1 in their flanks. Treatment was commenced two weeks after implantation when the tumors were about 20 mm³. After three weeks of treatment parthenolide and docetaxel as single agents both suppressed tumor

growth by 15% and the combination of docetaxel plus parthenolide decreased growth by 55% compared with control ($P = 0.01$) (Figure 6). The number of mice alive by day 43 decreased because the veterinarians declared the mice with large necrosing tumors were to be sacrificed. The number sacrificed were: control group 7 of 22; parthenolide alone group 3 of 20; docetaxel alone group 5 of 20 and in the combination 1 of 22 had needed to be sacrificed. A linear growth curve model was fit using a mixed model with a variance-covariance model that incorporates correlations of observations across time was also constructed. The rate of change of tumor volume between treatment groups was evaluated by comparing the slope estimates from the model. Data for this analysis was only taken out to day 22 of therapy because of the “drop-outs” from mice needing to be sacrificed. The rate of change for the docetaxel + parthenolide versus control was -43.11 ($p=0.0009$), and was also statistically less than docetaxel alone ($p=0.016$) and parthenolide alone ($p=0.011$). The rate of change for docetaxel versus control was -11.1 ($p=0.40$) and parthenolide versus control was -9.7 ($p=0.45$).

Small animal PET imaging using [¹¹C]CO and [¹⁸F]choline (FCH) was performed on a random subset of four mice from each group to assess *in vivo* blood volume and choline metabolism (cell membrane proliferation), respectively. The relative blood volume, expressed as the ratio of tumor surface to muscle [¹¹C]CO concentrations, was 1.31 ± 0.73 for solvent control; 1.27 ± 0.86 for parthenolide; 1.19 ± 0.29 for docetaxel and 0.50 ± 0.13 for the combination (combination vs. solvent control $p = 0.04$). FCH uptake was also significantly decreased in the combination group (control= 1.15 ± 0.10 ; D+P= 0.74 ± 0.24 , $p=0.05$).

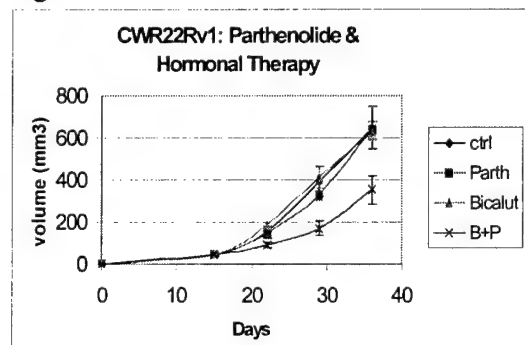
Interestingly, docetaxel, despite being at the forefront of current therapy for prostate cancer, is ineffective as a single agent in this animal model. The highly significant augmentation in docetaxel's efficacy when combined with parthenolide in the robust prostate cancer model

utilized in these studies is a very exciting result and illustrates the promise of this novel pro-apoptotic molecule.

Parthenolide with bicalutamide in an androgen independent prostate cancer heterotopic model:

To assess the ability to restore sensitivity to hormonal therapy we employed the same model as described for the parthenolide plus docetaxel. In this experiment, docetaxel was substituted with the anti-androgen bicalutamide at 50 mg/kg. This dose was shown to be the maximally effective dose in one of the hormonally sensitive derivatives of CWR22, (ie CWR22LD)¹⁷. After 21 days of therapy we observed that parthenolide can restore sensitivity to hormonal therapy as there is no inhibition with bicalutamide and with parthenolide

Figure 7:



After 14 and 21 days of therapy we observed that parthenolide can restore sensitivity to hormonal therapy as there is no inhibition with bicalutamide and with parthenolide after 14 and 21 days of therapy there was a 16% and 0% inhibition of growth respectively (ie minimal to no activity). However, the combination of bicalutamide plus parthenolide after 14 and 21 days

resulted in 57% and 45% inhibition respectively. Statistical analyses will be done as described above.

Using the C4-2 cell line in the tibia and measuring PSA six weeks, treatments were commenced and continued until week 10. The results of the serum PSA test are as follows:

Treatment	N	Average PSA (ng/mL)	SE	Number of mice with detectable PSA
Parthenolide	13	15.764	8.395	3
Parthenolide and Docetaxel	12	0	0	0
Control	16	4.756	2.595	4
Docetaxel	13	5.805	3.381	3

We saw that parthenolide had no effect as a single agent but was not different from docetaxel. The combination of parthenolide plus docetaxel had a profound effect with no mice having PSA at the time of sacrifice. The volumes of the tumors in the tibia were not measurable. This is another model that supports the data described above.

Finally our *in vivo* angiogenesis assay has shown parthenolide can decrease bFGF induced angiogenesis.

	VEGF OD - SE (%Inhibition)	bFGF OD - SE (%Inhibition)	Plasma Parthenolide concentration 1 hr after oral gavage
Control	4.55 - 0.37 100%	3.79 - 0.25 100%	0 µM
Parthenolide 0.4 mg/kg	3.89 - 0.23 14% (P=0.29)	3.00 - 0.22 21% (P=0.07)	Undetectable
Parthenolide 4 mg/kg	4.01 - .27 12% (P=0.46)	2.26 - 0.24 41% (P=0.0002)	0.112 µM
Parthenolide 40 mg/kg	3.60 - 0.26 21% (P=0.09)	2.08 - 0.30 45.3% (P<0.0001)	0.169 µM

Work to be done in the final year

- 1) parthenolide + bicalutamide in CWR22Rv1 *in vitro*. We initially planned to perform this experiment using C42 cells as the hormone independent cell line with charcoal stripped media inducing growth factor deprivation as a surrogate for "hormonal therapy". However, having gained access to the CWR22Rv1 cell line, we are able to use bicalutamide and hence perform a more clinically relevant experiment.
- 2) parthenolide + 2ME2 *in vitro* for HUVECS and CWR22Rv1 and *in vivo* for CWR22Rv1.
- 3) parthenolide + docetaxel *in vitro*.
- 4) immunohistochemistry evaluation of resected tumors (slides have been stained and are to be reviewed in March 2004).

Task 4. Evaluate the clinical relevance of NFκB activation.

a. Access paraffin embedded tissue from the pathology department of Indiana university Medical Center and stain for the p65 subunit and correlate with clinical outcome(Months 33 to 36).

Clinicopathological Experiments:

Ninety seven cases of radical prostatectomy were analyzed by immunohistochemistry. The antibody employed was a rabbit polyclonal antibody that identifies the binding site of the p65 subunit of the Rel family. (Santa Cruz Biotechnology, Santa Cruz, CA). Serial 5-μm-thick sections of formalin-fixed slices of radical prostatectomy specimens were used for the studies. The tissue blocks containing the highest Gleason score and the maximum amount of tumor were selected. Immunoreactivity for the p65 subunit was seen in 90%, 99% and 100% of all the specimens. However, the amount of immunoreactivity was greater in the neoplastic lesions compared with the benign glands. Specifically, the mean percent staining intensity was 14.7% in the benign glands and 79.2% in PIN and 88.7% in the adenocarcinoma. The difference in amount of staining was significantly greater in the PIN lesions compared with the benign glands ($p < 0.001$) and was greater in the invasive neoplastic disease compared with the intraepithelial neoplasia ($p < 0.001$)

Staining	Proportion p65 Present	Mean Staining (SD)	Range
Normal	90%	14.7% (17.1)	0-50
PIN	99%	79.2 (17.8)	0-95
Cancer	100%	88.7% (12.1)	35-95

Intensity	0	1	2	3
Normal	9(9%)	57(58%)	31(32%)	0(0%)
PIN	1(1%)	10(10%)	62(64%)	24(24%)
Cancer	0(0%)	0(0%)	29(29%)	68(70%)

The differential staining (frequency and amount) and graded increase between the normal, PIN and cancerous areas supports the contention that staining for the IκB binding site on the p65 subunit does represent activated NFκB. The presence of staining in all the cancer specimens support the *in vitro* data that activated NFκB is a common finding. The low amount of nuclear staining is probably due to the very short half life of transcription factors in the nucleus. The lack of association between pathological and clinical prognostic features is secondary to the universal staining at either 2+ or 3+ intensity.

This task has been completed.

KEY RESEARCH ACCOMPLISHMENTS

- NF κ B DNA binding is present in all prostate cancer cells and is overexpressed in human prostatectomy specimens
- Parthenolide decreases NF κ B DNA binding and many genes under its transcriptional control in both prostate cancer cells and endothelial cells.
- Parthenolide has single agent anti-cancer activity *in vitro* and can augment the efficacy of docetaxel
- *In vivo* parthenolide is able to restore hormone sensitivity to androgen resistant cell line
- Parthenolide has anti-angiogenic properties both *in vitro* and *in vivo*

REPORTABLE OUTCOMES

Manuscripts:

- "Nuclear Factor Kappa B is Constitutively Activated in Prostate Cancer *In Vitro* and is Increased in Prostatic Intra-epithelial Neoplasia and Adenocarcinoma of the Prostate".
- Submitted April 2003 to Clinical Cancer Research, received back 10/03, replies to reviewers comments resubmitted Feb 2004

Abstracts:

- Proceeding American Association for Cancer Research 2004
 - o "IN VIVO ANTI-PROSTATE CANCER PROPERTIES OF PARTHENOLIDE CORRELATED WITH PET IMAGING"

Presentations:

- none

Patents and licenses applied for and/or issued:

- patent application under review for use of parthenolide to treat cancer.
 - o **Patent number: 20030125373**

Funding applied for based on work supported by this award;

- Rapid access to intervention development – for purification and toxicity analysis with a goal to getting the drug to clinical trials, submitted February 2004
- DOD Idea Award: A mechanistic evaluation of parthenolide's anti-cancer properties – submitted February 2004

CONCLUSIONS:

We have shown that NF κ B is present in prostate cancer cells *in vitro* and in all clinical specimens of prostate cancer. This supports the notion that this is a relevant transcription factor to target for the treatment of prostate cancer. We have verified that parthenolide can inhibit NF κ B DNA binding *in vitro* and is orally bioavailable and able to inhibit angiogenesis *in vivo* and augment the efficacy of docetaxel and restore sensitivity to anti-androgens *in vivo*. This is at least partially due to parthenolide's ability to inhibit genes under the control of NF κ B. These findings provide strong supporting data to develop parthenolide as a new agent for the treatment of prostate cancer. Moreover, parthenolide is relevant as it has the potential to be a chemoprevention agent (because of the increased expression of NF κ B in PIN), and has anti-prostate cancer activity as a single agent and can augment the effectiveness of hormonal therapy, chemotherapy and radiation. All three of these modalities are very important in the treatment of prostate cancer. The ability of parthenolide to enhance their efficacy is therefore very relevant as it may in some cases save lives and in others prolong a patient's hormone therapy induced remission and hence their survival. An extensive amount of work is required to validate these claims.

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APPENDICES

None.